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Final report of the land-based testing of the AQUATRICOMB Ballast Water Treatment System (AQUAWORX GmbH), for Type Approval according to the Regulation D-2 and the relevant IMO Guideline

(April – July 2010)

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FINAL REPORT OF THE LAND-BASED TESTING OF THE AQUATRICOMB BALLAST WATER TREATMENT SYSTEM (AQUAWORX GMBH), FOR TYPE APPROVAL ACCORDING TO THE REGULATION D-2 AND THE RELEVANT IMO GUIDELINE

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1 Executive summary

The AquaTriComb™ BWTS, a ballast water management system, was tested according to the standard as documented in regulation D-2 of the "International Convention for the Control and Management of Ships' Ballast Water and Sediments," hereafter referred to as the "D2-Standard" and consistent with the IMO Guidelines for Type Approval testing (G8). The tests were conducted in the spring and early summer of 2010 in the harbour of the Netherlands Institute for Sea Research.

In general the basic requirements for testing which concern water characteristics and abundance of organisms were met and in most test runs environmental conditions were far more challenging than strictly needed. The AquaTriComb™ BWTS is designed as a UV-light disinfection system (LP-UV-reactor) with a self-cleaning filter (20 micron mesh size) as a primary treatment step, to reduce the number larger (planktonic) particles. The water is treated at intake and at discharge, i.e. a two-fold disinfection of the ballast water including a filtration step. The back flush of the self-cleaning filter at discharge was disinfected in the first UV-reactor receiving a high dosage UV-C radiation to ensure maximum mortality

At both salinities tested at least 5 test runs in a row were successful for all relevant parameters. During the combined test runs V and VI a sealing of the active filter was damaged. The damaged sealing was replaced after discharge of test runs V and VI. This allowed larger zooplankton organisms to pass the filter and enter into the system during these tests. These organisms were not inactivated completely by the UV-reactor during the 5 days holding period.

The sediment load was reduced in a pre-treatment step (self-cleaning mesh filter; 20 micron) and the remaining organisms were deactivated by ultraviolet light disinfection (three Low Pressure UV reactors in series, emitting UV with a wavelength of 254 nm).

Despite the fact that the numbers of phytoplankton were reduced immediately after the ultraviolet light disinfection step not all cells were fully disintegrated. Complementary tests indicated that a fraction of the remaining cells possessed a reduced viability or were only partly deactivated. Additional experiments showed a delayed mortality response of UV-radiation of up to three days in phytoplankton.

Treated discharged water, which was transferred to favourable growth conditions for plankton, showed no regrowth within a period of eight days. Environmental acceptability tests however showed that the growth of organisms, mainly plankton, was not limited by the discharge water indicating that the discharged water was still vital.

2 Zusammenfassung

Das AquaTriComb™ BWTS zur Behandlung von Ballastwasser wurde im Frühjahr und Sommer 2010 gemäß der Regularien-D2 (D2-Standard), sowie der IMO Richtlinien über Tests für eine Typzulassung (G8) im Hafen des Königlich Niederländischen Meeresforschungsinstituts (NIOZ) getestet. Generell wurden die allermeisten Anforderungen bezüglich der abiotischen Parameter des Testwassers und zur Organismendichte erfüllt. In den meisten Tests waren die Bedingungen sogar schwieriger, als in den Richtlinien verlangt. Das AquaTriComb™ BWTS arbeitet mit UV-Licht als Desinfektionseinheit (LP-UV-Reaktor). Zusätzlich verfügt es über einen mechanischen Filter (20 Micrometer Porengröße) als Vorabreinigungsstufe. Das Wasser wird bei Aufnahme und Abgabe behandelt, d.h. es findet eine Doppelbehandlung des Ballastwassers statt. Das Rückspülwasser des Filters beim Ablassen des Wassers wurde im ersten Reaktor einer erhöhten UV-C Dosis ausgesetzt, um die größtmögliche Abtötungsrate zu erreichen. Bei beiden getesteten Salzgehalten wurden mindestens fünf aufeinadnerfolgende Testläufe erfolgreich absolviert. Während der kombinierten Testläufe V und VI ging eine Dichtung des Filters kaput. Die Dichtung wurde nach Beendigung der Testläufe V und VI, d.h. nach dem Ablassen des Testwassers, ausgetauscht. Dies erlaubte größeren Zooplanktern in das System zu gelangen, welche durch den zweiten Behandlungsschritt mit UV nicht vollständig abgetötet werden konnten.
Die Sedimentfracht wurde in einem Vorbehandlungsschritt reduziert (selbstreinigende Automatikfilter und die Organismen durch UV-Bestrahlung inaktiviert (drei UV-Reaktoren in Serie mit Niederdruck-Lampen, Wellenlänge 254 nm).
Obwohl die Anzahl von Phytoplankton und Bakterien durch die UV-Behandlung stark reduziert wurde, wurden nicht alle Zellen vollständig zerstört. Ein Teil dieser Zellen zeigte noch eine eingeschränkte Vitalität und war nur teilweise inaktiviert. Ergänzende Versuche ergaben, dass Phytoplanktonorganismen eine verzögerte Mortalität, bis zu drei Tagen nach der UV-Behandlung zeigten. In Inkubationsexperimenten wurde über einen Zeitraum von 8 Tagen unter vorteilhaften Umweltbedingungen kein Planktonwachstum im behandelten Wasser festgestellt. Gleichzeitig durchgeführte Umweltverträglichkeitstests (Zugabe von Zeigeorganismen und Planktonkulturen, Verdünnungsreihen) mit dem behandelten Wasser ergaben jedoch keine negativen Auswirkungen auf das Planktonwachstum und zeigten somit, dass das behandelte Wasser an sich das Wachstum gesunder Organismen nicht beeinflusst.

3 Summary table with results for the Type Approval Certificate of the AquaTriComb™ BWT System

Land-based tests NIOZ	Reference & Treated			Reference			Treated			
salinity 23.8 PSU	**Intake**			**Discharge**			**Discharge**			**Discharge***
natural plankton	Average	min.	max.	Average	min.	max.	Average	min.	max.	Average
total bacteria [counts/mL]	5.81+E6	3.61-+E6	7.78+E6	4.41+E6	2.37+E6	8.19+E6	3.17+E6	2.07+E6	5.07+E6	1.91+E6
E. coli [cfu/mL]	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	<0.1
Enterococci [cfu/ 100mL]	< 1	< 1	< 1	< 1	< 1	<1	< 1	<1	<1	<1
plankton <10 µm [counts/mL]	5026	2118	8314	505	160	979	<10	<10	<10	<10
plankton 10-50 µm [counts/mL]	1391	1021	1897	138	110	204	<10	<10	<10	<10
plankton >50 µm [counts/m^3]	8.3+E5	1.2+E5	15.98+E5	2.15+E4	0.15+E4	4.24+E4	2.43	0.3	6.3	-

Land-based tests NIOZ	Reference & Treated			Reference			Treated			
salinity 35.3 PSU	**Intake**			**Discharge**			**Discharge**			**Discharge***
natural plankton	Average	min.	max.	Average	min.	max.	Average	min.	max.	Average
total bacteria [counts/mL]	8.89+E6	7.51+E6	11.4+E6	3.64+E6	3.17+E6	4.10+E6	3.64+E6	2.39+E6	6.83+E6	2.45+E6
E. coli [cfu/mL]	< 0.1	< 0.1	0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Enterococci [cfu/100 mL]	< 1	< 1	< 1	< 1	< 1	<1	< 1	<1	<1	< 1
plankton <10 µm [counts/mL]	5029	1943	9529	658	472	9529	<10	<10	<10	<1
plankton 10-50 µm [counts/mL]	1228	714	1650	100	87	120	<10	<10	<10	<10
plankton >50 µm [counts/m^3]	3.7+E5	1.7+E5	14.9+E5	3.57+E4	3.35+E4	3.84+E4	2.61	0.7	7.3	-

Summary table of collected data covering the major groups of organisms at two series of test-runs for low and high salinity range, respectively.
n.d.: non detectable in sample. **-**: no data; * viable cell as determined in bottle incubations of discharge water.

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5 Introduction

Ships transport 5-10 billion tons of ballast water annually all over the globe (Endresen et al. 2004). The ballast water is loaded with particulate sediment and an enormous variety of (living) organisms, which ranges from juvenile stages, larvae and eggs of fish and larger zooplankton (Williams et al. 1988; Carlton & Geller 1993) to macro algae, phytoplankton (Hamer et al. 2000; Hallegraeff et al. 1997), bacteria and viruses (Gollasch et al. 1998). In general these organisms belong to the natural ecosystem in and around the port of origin but they might not be occurring naturally in the coastal waters and port of destination at the end of a ship's voyage. In hundreds of cases around the world, this has resulted in severe damage to the receiving ecosystem and to human health, because these non- indigenous organisms developed into a plague. This often has a high impact on the natural ecosystem and can cause significant ecological and economical damage (Hoagland et al. 2002), as it results in a decrease of stocks of commercially valuable fish and shellfish species and occasionally outbreaks of diseases such as cholera (Ruiz et al. 2000; Drake et al. 2001). If action is not taken, the problem of invasive species will increase in an exponential manner for several reasons. Ships are getting larger, faster and the amount of traffic across the oceans is expected to increase rapidly during the coming decades. This results in an increased the volume and transfer rate of ballast water and therefore also the chance of non-indigenous organisms to have large enough numbers for settling and expanding. Our effort to reduce pollution of ports and coastal waters also improves the quality of the aquatic environment in these areas and therefore increases the susceptibility to invasive organisms. Originally not intentionally meant but organisms in ballast water will experience favourable conditions for settling and growing. The problem of invasive species is considered as one of the 4 major threats of the world's oceans next to land-based marine pollution, overexploitation of living marine resources, and physical alteration/destruction of habitats

To minimize these risks for the future, the International Maritime Organization (IMO) of the United Nations has adopted the Ballast Water Convention in 2004 (Anonymous 2005). The Convention states that ALL ships (>50,000 in number) should install proper ballast water treatment (BWT) equipment on board between 2009 and 2016. At present the number of countries ratifying the Convention is close to the required minimum and the expectation is that the Convention will be implemented in 2012.
An excellent overview of the current status and upcoming challenges for the implementation is given in the proceedings of the IMO-WMU Research and Development Forum of the meeting held in Malmö (Sweden, January 2010)
As a temporary and intermediate solution for the time being ships may reduce the risk of invasive species by performing ballast water exchange during their voyage when

passing deep water (>200 m depth and 200 NM from the coast. Ballast water exchange faces many problems as to feasibility, safety and efficacy. For a large part of ships' voyages the required depth and/or distance to shore requirements are never met; BW exchange can affect the ships construction stability and in rough seas exchange is not possible because of the risk to ship and crew. Treatment of ballast water is therefore considered to be the best solution of reducing the risk of invasive species.

During the recent years numerous solutions for treatment of ballast water have been mentioned and tested with the ultimate goal to reduce the amount of organisms in ballast water (Rigby & Taylor 2001). Treatment technologies based on UV-radiation are among the more successful ones at present. However, next to a high efficacy there is more needed for a BWT system to be a good solution to reduce the spread of invasive organisms. Next to biologically effective the system should be practicable, environmentally acceptable and also cost effective.
Despite the fact that the treatment technology for drinking-, waste- and process water is well-developed none of these techniques is directly applicable to ballast water (Rigby & Taylor 2001; MEPC 49/2/13, 2003). Besides reducing the load of organisms the sediment load should be reduced as well. There are also considerable differences in ships operation, types of ships, and the amount of space available for a ballast water treatment system on board and the way ships are operated. Ballast water treatment will develop into a new field of technology of its own with a commercial market estimated for the next 10 years in the order of 8 billion Euro (Tjallingi, 2001). Recent estimates indicated that the market may be even much higher (25 billion euro).

As a primarily scientific research institute NIOZ is defining its role in the certification process as to study:

1) the **numerical** abundance and biodiversity of organisms prior, during and after a treatment with the AquaTriComb™ -BWT system (efficacy of the BWT system),
2) to determine the **viability** status of the remaining organisms during discharge,
3) to assess possible **environmental risks** related to the use of UV disinfected ballast water by measuring residual effluent toxicity on planktonic organisms in order to determine latent effects, other than measured in specific toxicity tests conducted for the G9 (environmental impact).

This research strategy allows for more in depth testing, while it includes ALL organisms and not only the size classes as specified in the D2-Standard of the IMO Convention.

6 Description of the treatment facility

6.1 NIOZ Royal Netherlands Institute for Sea Research

NIOZ Royal Netherlands Institute for Sea Research is the National Oceanographic Institute of the Netherlands. NIOZ is part of the Netherlands Organization for Scientific Research (NWO). The institute employs around 250 people and the annual budget is approximately €25 million.
The mission of NIOZ is to gain and communicate scientific knowledge on seas and oceans for the understanding and sustainability of our planet. The institute also facilitates and supports marine research and education in the Netherlands and in Europe.

In order to fulfil its mission, the institute performs tasks in three specific fields.

Research: The emphasis is on innovative and independent fundamental research in continental seas and open oceans. The institute also carries out research based on societal questions when this merges well with its fundamental work. The senior scientists at NIOZ all participate in international research projects.
Education: The institute educates PhD and other students of universities and schools for professional education. Together with universities NIOZ also organises courses for PhD students and master students in the marine sciences. A number of our senior scientists of NIOZ is also appointed as professor at Dutch and foreign universities.
Facilitary services: NIOZ invites marine scientists from Dutch and foreign institutes and universities to write scientific proposals involving the institute's research vessels, laboratories, and the large research equipment, which is often designed and built by the institute's own technical department.
The basic oceanographic **disciplines** studied at NIOZ are physics, chemistry, biology and geology. Multidisciplinary research is regarded as one of the main strengths of NIOZ.

More information on www.nioz.nl

Figure 1: aerial view of the NIOZ harbour (lower right), NIOZ laboratories (upper left) and TESO ferry (top).

6.2 North Sea Ballast Water Opportunity project

From 2009 onwards the activities of NIOZ in ballast water research has been organized in broader framework, namely the North Sea Ballast Water Opportunity project (www.NorthSeaBallast.eu). This project was an initiative of the BSH (Federal Maritime and Hydrographic Agency, Germany) and the Royal Netherlands Institute for Sea Research (NIOZ, Netherlands), involves all relevant stakeholders within the maritime

sector in the North Sea region –governmental institutions, inter-governmental and non-governmental organisations, industry and scientific and technological institutes. This structure and participation offers a broad and sound base for the project in support of a succesful implementation of the IMO Convention in the region, as North Sea, Skagerrak, and Kattegat form one ecological zone. Moreover, the project being one of the largest and most integrative in its kind, the objectives (investments) will become available as a model for other european maritime regions as well as across the globe. To facilitate this initiative funding was received from the North Sea InterregIVB (an ERDF program). For the embedding in a more global strategy the project is liaising with the Globallast II initiative of the IMO and currently involves also comparable research initiatives in the US (GSI, MERC and Golden Bear).

6.3 Portrait of Aquaworx (producer of the Aqua TriComb™ ballast water management system)

More information on: www.Aquaworx.de

Aquaworx ATC GmbH, www.Aquaworx.de, founded in 2008, has developed proprietary ballast water treatment systems that have been specifically designed to effectively, environmently friendly and economically eliminate the worldwide transfer of aquatic invasive species.

The Aquaworx ATC GmbH has its headquarters in Munich, Germany and was founded in October 2008. Mr. Peter Falk, an experienced industry and sales professional, is leading the Aquaworx ATC GmbH as managing director. Aquaworx markets its solutions worldwide from Germany using industrial partners and sales organizations. The technical development of diverse Aquaworx products began in 2005. The systems are basically working according to the principle of combining UV light with ultrasound. In April 2008 the decision was made to step into the ballast water treatment field.

Aquaworx ATC GmbH is in collaboration with the Royal Netherlands Institute for Sea Research (NIOZ) to conduct certification testing also together with the German Federal Maritime Agency (BSH), who will act as the administrating agency during the certification and Type Approval process.

6.4 The test facility

The land-based tests were carried out at the Royal Netherlands Institute for Sea Research (NIOZ), Landsdiep 4, 1797 SZ 't Horntje, Texel, the Netherlands, from March through July 2010 (www.nioz.nl, figure 1).

The NIOZ harbour test site is equipped with 3 coated tanks of 300 m³ volume each to simulate the ballast water tanks of the ship (Figure 2). The tanks were (high pressure steam) cleaned after each run. Water samples can be taken from bypasses of the standard piping (DIN 200) used to fill and to empty the tanks.

Figure 2:
Three sample tanks

According to the requirements of the Guidelines G8, sampling points are fitted before the treatment system and directly after the system. Samples varying in volume from 1 L up to 1 m^3 (IBC's) were taken using clean sampling containers. Sampling containers and all further handling of the samples were separated in a control and a treated set to avoid cross contamination by any potentially active substance. The basic handling, such as concentrating, filtration and chemical analysis was done at the test site. Different samples (1 to 10 L) were transported to the institute's laboratories for further special analysis. For re-growth experiments 10 L of sample was transported (Nalgene bottle) to a climate room for incubation experiments (ca. 12 – 15 °C; a light;dark regime of 16:8 h and 100 μmol quanta. $m^{-2}.s^{-1}$)

The Aqua TriComb™ is connected to a water pump (capacity of up to 500 m^3/h) which was located in the NIOZ harbour. This is a pristine harbour with a direct access to the Wadden Sea and the origin of the test water changes with the tide. Furthermore, provision were made to allow the addition of brine water and / or freshwater in order to adjust the salinity of the natural water of the NIOZ harbour to the required test conditions of brackish water and marine water with a minimum of 10 PSU difference.

Figure 3:
Water pump with self suction installation (based on vacuum)

A detailed description of the test installation is presented in figure 4.

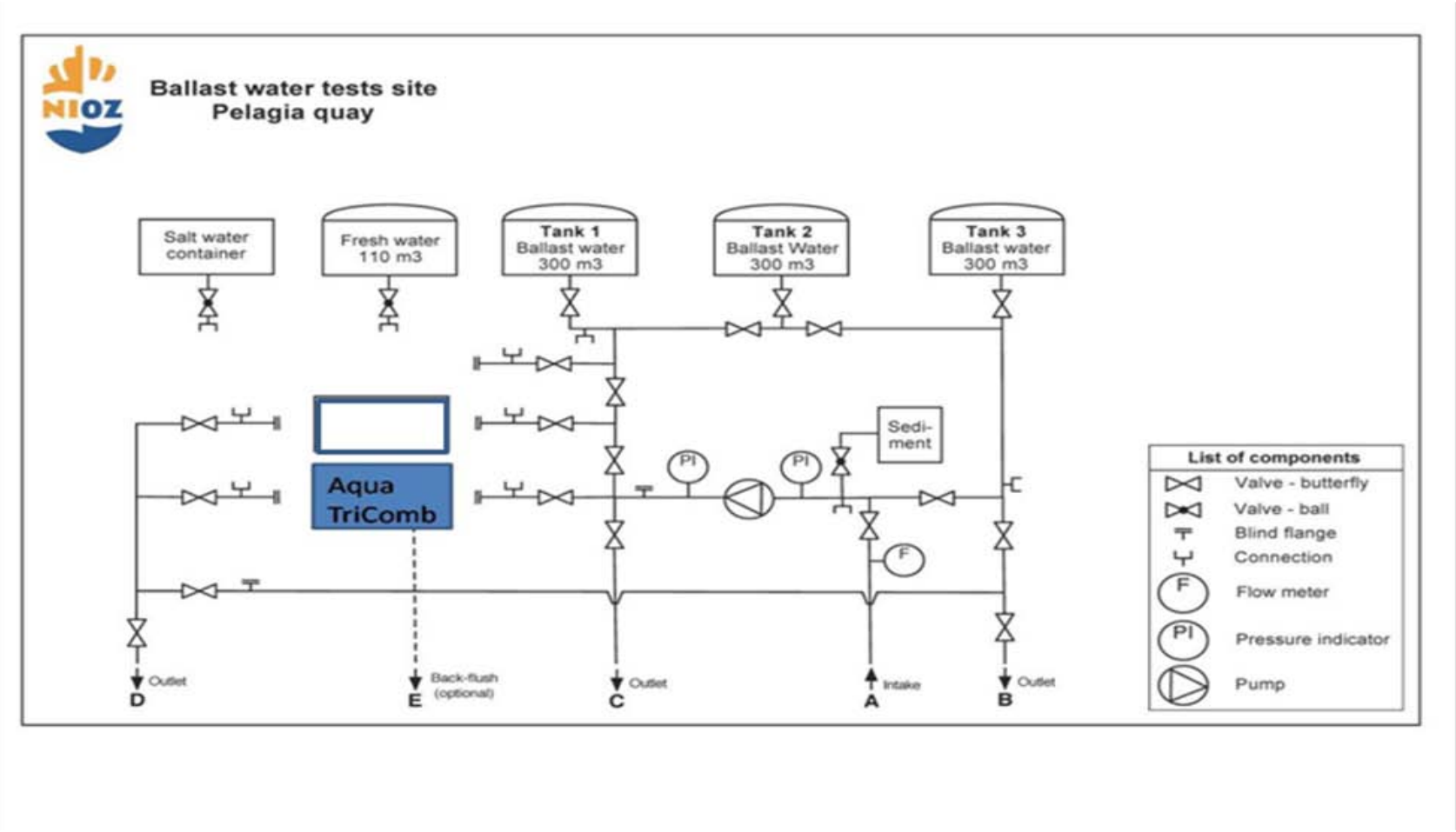

Figure 4: P&I diagram of the Aqua TriComb™ installation at NIOZ with points of intake, outflow back flush and sampling

6.5 Technical description of the Aqua TriComb™ System

Aquaworx ATC GmbH has developed the ballast water treatment system AquaTriComb^{TM} comprising pre- and after treatment modules. The capacity of the volume flow rate can be designed by means of aligned up-scaling of the modules.

The AquaTriComb^{TM} BWTS comprises mechanical filtration with a self-cleaning suction process as the pre treatment step. The after-treatment compose ultraviolet-C (UV-C) radiation in combination with ultrasound (US) for self-cleaning of UV-C sleeves.

Due to the highly efficient combination of US oscillation for cleaning and UV radiation for disinfection the AquaTriComb^{TM} technology can refrain from any preparations or chemical additives, which may pose a hazard to human health and / or to the environment.

Because the US and UV-C technology is precisely adjusted to avoid the generation of OH• radicals, the concentration of the OH• radical and other related Other Chemicals (acc. to G9) is that low that risks to human health and to the environment can be demonstrably excluded (see risk assessment analysis in submission for Basic Approval, MEPC 59/2/8).

During ballast water intake, the pre-treatment stage for removal of particles and organisms, the ballast water is filtered through one MicroSintFilter (MSF) module. All elements of the filter cleaning technology are developed by Aquaworx. The filtration technology with 20 µm pore size allows very effective removal of particles and organisms. The automatically self-cleaning filter process is based on permanent suction technology. Filtration process is designed as one filter is able to produce permanently 250 m³/h treated ballast water. During the production stage of one filter, the other identical filter is in stand-by mode.

After the filtration stage, the water is exposed to monochromatic UV light inside the UV-reactors. The low pressure mercury germicidal lamps produce UV-C light at a wavelength of predominantly 254 nm. Cleaning of the lamp protecting quartz sleeves is done periodically by using low frequency ultrasound. The application of low pressure UV-technology at 254 nm wavelength disinfects water containing aquatic organisms very effectively without generating any chemicals.

The most important impact of the ultrasound is the highly effective cleaning of the UV-Quartz sleeves through the ultrasonic transmission along the full length of the UV-reactor, avoiding accumulations of bio films and/or inorganic salts on the quartz sleeves, assuring that the produced UV light is able to penetrate the water to achieve the required killing rate of the organisms. During the disinfection treatment inside the UV-reactor the organisms are lethally affected by UV-light.

During de-ballast operations the ballast water from the tanks is again treated by the complete pre- and after treatment plant before discharge. Organisms, e.g. bacteria and monocellular heterotrophic plankton, which in spite of effective treatment may grow again during long ship voyages, will be effectively killed. Backwash water is treated during a special developed process. During filtration of de-ballast water the backwash is not discharged over board. The backwash is treated by the first UV reactor. Because of the low flow rate the backwash water has a relative long residence time in the UV reactor, which results in a treatment with a high UV dose. This results in an extremely effective treatment through the high energetic impact of UV radiation. After the special treatment of the backwash water it is added to the main filtration stream.

Figure 4: Aqua TriComb™ ballast water treatment system. Inside view of test container.

The system consists of three UV-reactors, a Low-Pressure ultraviolet light disinfection system with a typical output of UV-C (Fig 5; 254 nm wavelength) combined with a pre-filter. The advantage of a pre-filter is to reduce the number of larger organisms of which is known that UV-radiation is not a very effective way of deactivation (Raikow et al. 2007). In addition filtration reduces the load of (larger) particles and increases therefore the transparency of the water, hence the effectiveness of the UV-reactor.

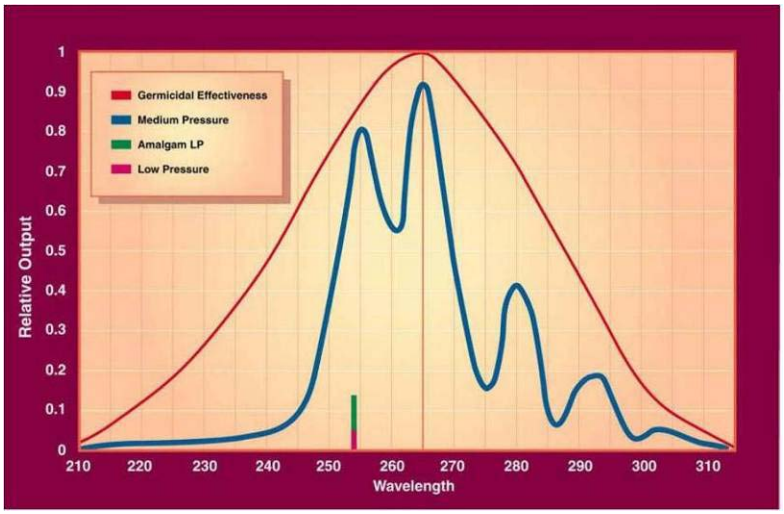

Figure 5: spectral composition of medium- and low-pressure lamps.

Unlike active substances the working of ultraviolet radiation (UV-C) is based on transferring electromagnetic energy to molecular components of organisms, resulting e.g. in DNA damage. This will prevent cell replication, and therefore growth, but also causes (immediate) disintegration of cells, morphological changes, discoloration, cellular membrane leakage and cell damage.

The end of lamp life is not when a lamp fails to light but rather when its output has decreased by 10%. The Aqua TriComb™ uses a UV sensor to continuously monitor the

output of the lamps. Besides monitoring the output of UV-C (254 nm) the UV sensor can also monitor the cleanliness of the sleeves, aging of the lamps and indirectly the water quality. In addition the Aqua TriComb™ monitors the produced amount of UV light (UV intensity) per individual UV reactor for better observation of disinfection process

Because the power delivered to the UV fluorescent lamp is not adjustable the majority of the time the system will be overdosing the UV. This is probably the only disinfection technology that can safely be overdosed. A new lamp will be running about 130% of the required UV-radiation.

The Aqua TriComb™differs from other BWT-systems as the system is not only treating during intake (mesh filter and UV-reactor) but also at discharge (mesh filter and UV-reactor). The back flush at discharge is treated in a separate UV-reactor where the (remaining) organisms receive an extra high UV dosage. Assessment of the efficacy of the BWT system was therefore immediately at intake (T0), at discharge after a holding period of 5 days (T5) and 7 days after discharge (T12)

The applied test protocols (Anonymous, 2010) were communicated with the German Administration (Federal Maritime and Hydrographic Agency of Germany; BSH) and a brief description of the various methods is included in the next section. During the certification process the whole practical procedure of intake and discharge has been witnessed on one occasion by the national administration.

7 Requirements to meet the D2-Standard

According to the D2-Standard of the IMO/MEPC Convention of 2004 (Anonymous 2005) ships that meet the requirements of the Convention by meeting the ballast water performance standard must **discharge**:

> ***1) Less than 10 viable organisms per cubic metre greater than or equal to 50 micrometers in minimum dimension;***
>
> ***2) Less than 10 viable organisms per millilitre of less than 50 micrometers in minimum dimension and greater than or equal to 10 micrometers in minimum dimension and***
>
> ***3) Less than the following concentrations of indicator microbes, as a human health standard:***
>
> ***3a. Toxicogenic Vibrio chlorerae (serotypes O1 and O139) with less than 1 colony forming unit (cfu) per 100 millilitres or less than 1 cfu per 1 gramme (wet weight) of zooplankton samples:***
>
> ***3b. Escherichia coli less than 250 cfu 100 millilitres;***
>
> ***3c. intestinal Enterococci less than 100 cfu per 100 millilitres.***

The present D2-Standard is defined as a standard for the water characteristics at **discharge**. Furthermore, with exception of some indicator microbes (point 3) organisms < 10 µm are completely excluded. This should certainly be considered as an omission since this size class in particular includes numerous phytoplankton species characterized as Harmful Algal Blooms (HABs).

Nevertheless, the D2-Standard is clear with respect to the maximum number of organisms remaining present. On the other hand a proper definition of the dimensions of organisms is still subject of (academic) discussion. Moreover, as an operational definition for viable organisms the IMO is using: "*organisms and any life stages thereof that are living*", but a more adequate (scientific) definition is: an organism that is able to complete it's life-cycle, including reproduction (DNA replication). In the case of UV it is known that for certain organisms the cells will be (DNA) damaged but remain active for a period of up to several days. The organisms however, lack the ability to reproduce. This is likely to happen considering the type of UV irradiation (Low Pressure, 254 nm wavelength) applied in the present BWT system.

In addition to the (basic) requirements for the D2-Standard NIOZ has adopted a variety of methods and techniques to determine abundance, sizes and the viability status of different types of organisms also present in the various types of aquatic waters. This includes also plankton < 10 µm other than bacteria (i.e. phytoplankton and viruses). Moreover, we extended our research effort to examine not only the fate of the organisms in the large-scale holding tanks but also took subsamples for incubation under optimal growth conditions to study the growth potential of remaining (viable) organisms and/or survival stages such as eggs, cysts or dormant cells over a longer period than the recommended 5 days. These later experiments allowed us also to study potential latent toxicity effects (environmental acceptability).

7.1 Requirements to meet: guideline G8

Next to the D2-Standard two guidelines were developed by the IMO as a framework for approval of ballast water treatment systems (G8) and approval of the use of active substances in ballast water treatment systems (G9). For land-based testing MEPC 53/Annex 3 (Anonymous 2005) and modifications as adapted at MEPC 174.58 (Anonymous 2008) was compiled of which the most relevant parts will be presented below. These guidelines were generically designed to meet the conditions of a broad range of potentially effective treatment techniques to be tested in typical port and environmental conditions found across the globe. Most test protocols therefore require extensions of the test design to cover the specific aspects of the treatment. The land-based testing serves to determine the biological efficacy of the BWT systems under consideration for Type Approval under more or less controlled and replicable conditions. This is intended to ensure that the efficacy of the equipment is consistent and can be shown repeatedly. The test set-up should therefore be representative of the characteristics of the arrangements used and the type of environment the BWT system was designed for.

One of the main criteria in the G8 test requirements is the salinity range and related to this the differences in Total Suspended Solids (TSS), Particulate Organic Carbon (POC) and Dissolved Organic Carbon (DOC). This resulted in three main categories of test conditions (Table 1).

Table 1: Three different salinity ranges and minimum concentrations of TSS, POC and DOC in the water as indicated in G8 guidelines.

	Salinity			
Parameter	**> 32 PSU**	**3 – 32 PSU**	**< 3 PSU**	**unit**
Total Suspended Solids (TSS)	> 1	> 50	> 50	mg/L
Particulate Organic Carbon (POC)	> 1	> 5	> 5	mg/L
Dissolved Organic Carbon (DOC)	> 1	> 5	> 5	mg/L

In general UV-reactors are not sensitive to changes in the salinity of the water except that water with a high alkalinity, iron and/or manganese influences the transmittance of the water and can, depending on the UV sleeves cleaning technology, will increase fouling of the UV-light source (Gundry 2007). Historically UV-reactors are widely used in treating (fresh) drinking water and sewage water and its use as a tool for disinfection is

well studied. It was for this reason that the Type Approval tests were conducted at the intermediate (3 – 32 PSU) and high salinity (>32 PSU) regions. The difference in composition of the test water between the freshwater and intermediate salinity water is the presence or absence of (sea)salt. All other minimum requirements for TSS, POC and DOC for these two water types were identical (Table 1).
A further requirement is that the difference between the two salinity regimes should be at least 10 PSU. The test water, originating from the Wadden Sea, and the actual sampling did vary with the tide and as a result salinity was subject to variations. To assure the 10 PSU salinity differences it was decided to have the possibility of adding fresh water and upgrade coastal water of the North Sea water by enhancing the salinity (brine solution of commercially available sea salt; ca. 18%). As target number the freshwater addition was adjusted to a salinity of ca. 23 PSU for the low salinity regime and ca. 33 for the high salinity regime. In practice ca. 15 % (v/v) of freshwater was added during the low salinity tests and about 4% of brine solution (Instant Ocean®), during the high salinity test runs. In order to compensate the dilution of the TSS by the freshwater some extra sediment (taking from a nearby mudflat the Mokbaai) was added as well. These additions were made close at the pump site, to ensure proper mixing, with a constant flow rate and done during filling of the control and the treated ballast tank.

Biology

The guideline G8 also defines criteria for the number and diversity of the organisms to be met during Type Approval testing (Table 2). These criteria should be met for all three salinity regions.

Table 2: Minimal numbers and species diversity required at intake for different size classes and groups of organisms as indicated in G8 guidelines.

Influent test water		
Parameter	**unit**	**Remarks**
organisms ≥ 50 micron	> 10^5/ m^3	at least 5 species from at least 3 different phyla/divisions
10 ≤ organism size ≤ 50 micron	> 10^3/ mL	at least 5 species from at least 3 different phyla/divisions
heterotrophic bacteria	> 10^4/ mL	not further defined

The test water should contain minimum densities of plankton which are typical densities encountered in the Wadden Sea during the annual spring bloom in April/May. With respect to the species diversity, the Wadden Sea is known for its natural richness in organisms and during the test period (April – July) indeed a large diversity in organisms, adults, juveniles, eggs, etc. was encountered.
An important aspect, so far not recognized in the guidelines (G8), when dealing only with natural populations of organisms in the influent of the test water is the natural seasonality of species and blooms. The actual onset of the spring bloom is dominated by phytoplankton, but usually lacks high zooplankton abundance. Only at a later stage zooplankton starts to increase in abundance, subsequently due to predation it will diminish the numerical abundance of (smaller sized) phytoplankton component.
Furthermore, for the high salinity range, the composition of the organisms in the water resembles that of a typical oceanic environment. This implies an increase of smaller

sized cells, down to the micrometer scale, and also a dramatic decline in the number of larger (>10 µm) organisms.

Human pathogens

Table 3: Maximum allowed numbers of 3 groups of indicator microbes in the effluent test water on discharge as indicated in G8 guidelines. cfu: colony forming units

Effluent test water		
Parameter	**unit**	**Remarks**
Toxicogenic *Vibrio chlorerae*	< 1 cfu/100 mL or < 1 cfu/ g wet weight of zooplankton	serotypes O1 and O139
Escherichia coli	<250 cfu/ 100 mL	
intestinal Enterococci	<100 cfu/ 100 mL	

Within the group of prokaryotic microbes only bacteria and more specifically the heterotrophic group (Table 2) has been taken into account by the standard but for completeness this should include all bacteria and presently also Archea. While these microbes are part of the natural community in the aquatic environment the indicator microbes (Table 3), i.e. the human pathogens, are introduced as part of human activity and often associated with discharge of sewage. In the present research all microbes have been included as a bulk parameter, the number of heterotrophs as a viable component as well as the viability of the whole microbial community has been determined.
Within the whole microbial community the number of heterotrophic bacteria was determined as well as *E. coli* and total enterococci. The test area of the institute is part of a tidal estuary of the Wadden Sea, which is essentially a pristine environment. Moreover, waste water treatment is highly developed in the Netherlands. Therefore, numbers of these human pathogens during the tests were to be expected to be low for most of the sampling period. On the other hand during the different treatment steps a significant amount of particulate organic material is transferred into dissolved organic carbon (DOC) which acts as an excellent substrate stimulating growth of (heterotrophic) bacteria. The group of *V. chlorerae* is not present in the region; therefore no samples were taken to determine the presence of these pathogens.

7.2 Experimental design

A variety of methods was applied to examine the biological efficacy of the Aqua TriComb™ for the different categories of organisms during the two test series. For detailed description we refer to read the outline of the official test protocols for the Aqua TriComb™(Anonymous 2010). Sample handling and volumes were according to the description of the guideline for BWT testing (G8) or described in detail when these guidelines were insufficient or other considerations were taken into account, e.g. in the case of sampling and incubation of samples at discharge. Subsamples were taken at random or throughout the whole filling procedure of the tanks. As indicated previously there was great emphasis on analysing of the freshly taken samples and having multiple methods to examine numerical abundance and viability of the organisms present. Besides various biological samples there was also a basic set of physical and chemical

parameters which were monitored prior, during and after discharge. A short description of each parameter and how it has been analysed is given below.

Physical and chemical properties of test water

Temperature

The water temperature was measured using a calibrated thermometer.

pH

The pH-level is measured using a calibrated pH-meter.

Salinity

For salinity ca. 250 - 500 mL water is sampled and stored at room temperature (glass bottles) until analysis by direct measurement in the laboratory at NIOZ. Salinity of the water was measured after each test cycle using a field salinometer and a refractometer (Atago) calibrated against 0 and 33 PSU standard seawater. The accuracy of the salinity measurement is 0.5 PSU.

Dissolved Oxygen

The spectrophotometric method of the Winkler method (Winkler, 1888; Pai *et al.*, 1993); Reinthaler, 2006) was used to determine the oxygen concentration in the water. Samples were taken using gastight tubing which was specially fitted to the sampling tubing that was used to sample the ballast simulating tanks. The coded glass bottles are flushed at least three times their volume (ca. 120 mL) with water.

The sample bottles were stored in a dark container filled with water of the same temperature as the samples until further analysis at the laboratory. In the laboratory 1 mL H_2SO_4 is added prior to measuring the optical density (OD) at 456 nm with a Hitachi U-3010 Spectrophotometer. The oxygen concentration was calculated using standards and expressed as μM O_2/L (or mg O_2/L; = μM O_2 * 0.032). Since both salinity and temperature change over the season the oxygen concentration was expressed as percentage relative to the natural saturation value for the given temperature and salinity.

DOC

The concentration of dissolved organic carbon (DOC) were measured according to Reintaler & Herndl (2005). Samples for DOC (15 mL) were filtered through GF/C filters and sealed in pre-combusted glass ampoules after adding 50 µl of hydrochloric acid (HCl). Sealed ampoules are stored at 4 °C. The DOC concentration was determined in the laboratory by the high temperature combustion method using a Shimadzu TOC-Vcpn analyzer. Standards were prepared with potassium hydrogen phthalate (Nacalao Tesque, Inc, Kioto, Japan). The mean concentration of triplicate injections of each sample (three in total) is calculated. The average analytical precision of the instrument is < 3 %.

Figure 6: the challenge water with a high particle and sediment load.

TSS / POC (total suspended solids and particulate organic Carbon)

For TSS/POC pre-weighted glass fibre filters (Ø 47 mm, GF/C) were used. Each filter was coded and stored in a clean Petri dish. The filtered volume was dependent on the particle load and concentration and type of organisms present in the water. The higher the total particle load in the sample, the smaller was the volume that could be filtered before the filter clogged. Practical volumes were between 100 and 1000 mL per sample.
After filtration the filter was rinsed with fresh water (MiliQ) to remove sea salt. Filters were dried overnight at 60 °C and allowed to cool in a vacuum exicator before weighing. The total amount of suspended solids was calculated from the weight increase of the filter and averaged for the three replicates (mg/L).
Next, the filter is combusted at 500°C (overnight) and allowed to cool in a vacuum exicator and weighted again. The POC was calculated from the weight decrease between this measurement and the TSS weight.

Biology

The majority of the large size fraction (>50 µm) consists of zooplankton, while the majority of the small size fraction (10-50 µm) consists of phytoplankton. Organisms > 50 µm are retained as recommended in MEPC 54/Inf.3 (using a modified Hydrobios net).

Samples for the 10 - 50 µm fraction were collected as whole undisturbed samples. These samples were then filtered over a 50 and a 10 µm sieve and fixated.
A second set of samples for this size class was taken and not separated from the organisms < 10 µm in order to include the fate of the smaller sized (phyto)plankton community as well and to avoid further damage of the plankton. The results of these samples were compared to the ones from the double filtered samples to evaluate the loss of organisms caused by processing the samples.

Sample sizes

During the land-based tests containers from 1 to 1000 L were used for sampling and/or storage. Samples were taken continuously and sequentially during the whole process of filling or emptying the ballast water tanks. These containers were thoroughly rinsed or heat-treated prior to use. Samples for the human pathogens were taken in sterile (bar-coded) bottles provided by the bacterial test laboratory.

Figure 8: mud container and sample bottles (left), large IBC (1000 L) sample container (right).

Organisms > 50 µm

The samples are pre-concentrated over a Hydrobios 50 µm net resulting in an end volume of approximately 100 to 200 mL. The samples were transferred to the lab directly after sampling and Neutral Red was added in a ratio that yields an end concentration of 1:50,000. Staining time is 2+ hours. Neutral Red stains living organisms (Crippen& Perrier 1974; Fleming & Coughlan 1978) distinctively and quite rapidly (less than one hour, figure 9). Therefore the viability assessment remains unaffected by the possible death of organisms during the staining or during sample analysis.
It is assumed that dead but physically intact organisms will also be found. Consequently a detailed inspection of each intact individual is needed to assess viability. This includes the staining as well as the detection of internal (heart, gills) movement. Organisms which were not intact are assumed to be dead.

Neutral Red is a reliable staining method for all major groups of organisms but inconsistent staining was found for bivalves. For this latter group movement (including internal such as heart and gills for juvenile mussels) has to be used obligatorily to determine viability. For consistency and internal quality check samples were analyzed by qualified and well trained experts.

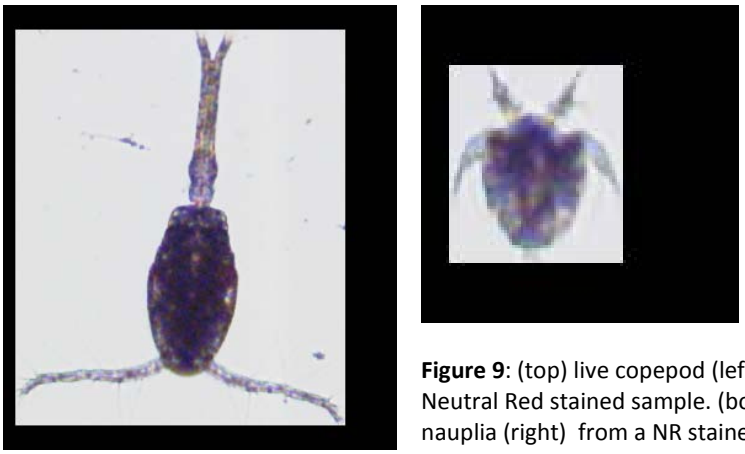

Figure 9: (top) live copepod (left) and nauplia (right) from a Neutral Red stained sample. (bottom) dead copepod (left) and nauplia (right) from a NR stained sample. Pictures made with the FlowCam.

The samples are analyzed manually using a binocular with a 20x magnification for counting and up to 50x for species identification and measurements when necessary.

For inter comparison a subset of samples was also analyzed using a semi-automated tool (Fig. 9; FlowCam, Fluid Imaging Technologies; Anonymous 2001).

Organisms < 50 μm

Samples for visual inspection of species number and diversity were pre-concentrated using a sieve made of a Hydrobios 10 μm mesh net using the 50 μm prefiltered sample (effective size range is 10 – 50 μm). The retained organisms were flushed into 50 mL Greiner tubes using filtered seawater and fixed with Lugols solution. Sample analysis was conducted by microscopic count with an inverted microscope at 200x magnification (method by Utermöhl). Since the Utermöhl is not suitable to asses viability counting was restricted to the structural integrity of organisms and therefore the presence of intact cells (Paerl 1978). This method works for both zoo- and phytoplankton.
The size fraction < 50 μm was also covered by flow cytometry on basis of a single cell measurement (Veldhuis & Kraay 2000) and PAM fluorometry, as a bulk parameter (Schreiber et al. 1993), using the intact and undisturbed samples (taken from 1 L sample bottles). Besides numbers and sizes these two methods can be used to assess the cell viability (Veldhuis et al. 2001; Veldhuis & Brussaard 2006) or in case of the PAM fluorometry also the photosynthetic efficiency of the phytoplankton.

Flow cytometry: For total organisms counts 3 mL of unfiltered sample water (reference and treated, each in triplicate) were analyzed using a calibrated flow cytometer. This yields the total number of particles (dead and live organisms as well as detritus) as well as their size range and the presence or absence of chlorophyll. For the counts exactly 1 mL was analyzed.
The size of the plankton was determined by comparison to standardized beads (10 and 50 μm). These beads were also used as standards to calibrate the performance of the flow cytometer.
For organism viability testing, on the level of the individual cell, SYTOX Green was added to 1 mL of sample water (control and treated, each in triplicate). After 15 minutes samples were analyzed using the flow cytometer for the presence of dead and/or life organisms (cf. Veldhuis et al, 2001, Fig. 10).

Figure: 10(A) Epifluorescence microscopic picture of a life phytoplankton cell. The red signal is due to the presence of chlorophyll and (B) a dead phytoplankton cell with a yellow/green fluorescence of the nucleus after staining with SYTOX Green.

PAM fluorometry: The photochemical efficiency of photosystem II of phytoplankton (providing an estimate of the general health of the algae) can be addressed using Pulse-Amplitude Modulated fluorometer (PAM-fluorometry) WALTZ- water PAM (Schreiber et al. 1993). For this 3 mL of unfiltered sample water (control and treated, each in

triplicate) are filled into a glass cuvette and analysed using the Pulse-Amplitude Modulated fluorometer. The instrument was calibrated against filtered seawater.

Bacteria

The classical method for counting bacteria in many applications is based on plating on selective media. Unfortunately, for studies in the aquatic environment this approach is by far insufficient for various reasons (Gasol and Del-Giorgio, 2000). As a result total bacteria were now determined by flow cytometry, using DNA-specific stains to get a more accurate bacteria number. In addition samples were taken at discharge for specific human pathogens and heterotrophic bacteria using a plate method.
For total bacteria number a 1.5 mL water sample was taken and pipetted in a Cryovial (in triplicate) and formaldehyde was added as a preservative. Samples were frozen and stored at -80 °C until further analysis.
Upon analysis the sample is allowed to thaw completely. A subsample of 100 µl was diluted with a TE-buffer, and the nucleic acid dye PicoGreen (MP) was added. Within 10 to 20 minutes after the addition of the stain the sample is analyzed using a flow cytometer (cf. (Gasol *et al.*, 2000; Veldhuis *et al.*, 1997). A known bacterial standard is used for calibration and counting.
The number of total heterotrophic bacteria was determined using a plate method as the number of colony forming units (cfu's) after incubation of the water at intake and discharge according to an international standard (NEN-EN-ISO 6222:1999 "Vitens laboratory bv" at Leeuwarden, RvA lab. no. L043)).

Human pathogens

The samples for microbiological analysis of the presence and number of human pathogens were taken in special bottles of 600 mL and send to "eurofins/C.mark bv" at Heerenveen (accreditation certificate: RvA lab. no. L043). All analyses were carried out according to NEN-EN-ISO standards.
These samples are sent to the laboratory immediately after sampling using a cooled transport container (4 °C). The analysis is carried out according NEN-EN-ISO 7899-2 for intestinal enterococci and NEN-EN-ISO 9308-1 for *E. coli* and related bacteria of the coli group as adopted for surface and waste water analysis in the Netherlands.

8 Results

The present section is a compilation of all relevant information needed for Type Approval Certification tests according to the Guidelines (G8), but also includes some relevant results of experiments conducted to assess the environmental acceptability of the applied UV-reactor and/or its potential by-products in the environment upon discharge. Data are presented as averages or ranges separated for the two salinity regimes tested. In Annex 1 a detailed species list of observed organisms is presented.

Figure 11: overview of the Aqua TriComb ™ Ballast Water Treatment System in the NIOZ harbour (top). Inside view of the ATC (bottom).

The tests were carried out at two different salinity regimes (Tables 4 and 5) hereafter referred to as low and high salinity test series.
It should be noted that two full scale treatments tests were conducted in series at every test cycle. After the first treatment test the whole BWT system was shut down according to the manufacturer's procedure. The control tank was filled (only pumping water in the tank) immediately after the first test run. The second treatment test run, starting with a complete starting up procedure, was after the control tank was filled. This means the two test runs shared the same control. While sharing an identical control the two treatment tanks were sampled independently.
In between complete test runs the filters of the Aqua TriComb™ BWT system were (high pressure) cleaned and system was rinsed with fresh water.

8.1 Physical and chemical parameters

Table 4: Average salinity and temperature of water at intake during the low salinity tests of control and treated tanks for test runs I – VIII.

test run	salinity [PSU]	range	temperature [°C]
I –VIII	23.8	23-25	7.2 - 9.3

To achieve a sufficient low salinity value during the test cycles brackish water was collected from the NIOZ harbour during low tide. Since the salinity was not low enough extra fresh water was added to a maximum of 15% (v/v).

Table 5: Average salinity and temperature of water at intake during the high salinity tests of control and treated tanks for test runs IX – XIV.

test run	salinity [PSU]	range	temperature [°C]
IX - XIV	35.3	33-36	9.9 - 12.2

To the high salinity test cycles coastal water from the North Sea was collected during high tide and a brine solution made from sea salt was added to a maximum of 4 % (v/v).

In regards to salinity, the difference between both the high and low salinity regimes was 11.5 PSU. This average value meets the criteria set in the G8 guidelines of a salinity difference of at least 10 PSU. It should however be noted that the efficacy of the Aqua TriComb™, which is based on a filtration and UV-reactor, is not affected by changes in the salinity within the salinity regime tested.

Low salinity

A summary of the results of the basic parameters (oxygen concentration, pH, TSS, POC, DOC and nutrients) is presented in table 6 for the reference and treated water sample at intake and discharge.

Table 6: average or range of oxygen, pH, inorganic nutrients, TSS, POC and DOC concentrations of test series run at low salinity (8 test runs in total; I-VIII) at intake and discharge; [1]: relative to saturation value for given salinity and temperature. For intake samples were collected just prior pumping water into the storage tank. In the case of treated water after the treatment system.

Parameter	Intake	s.d.	Discharge (day 5)	s.d.	Unit
O_2 reference[1]	100-106	-	74-89	-	%
O_2 treated[1]	96-116	-	85-101	-	%
pH reference	8.22	0.10	8.10	0.19	
pH-treated	8.22	0.10	8.16	0.13	
NO_3 reference	28.5	5.43	28.0	5.62	µM
NO_3 treated	29.2	4.85	29.0	4.9	µM
NO_2 reference	0.54	0.07	0.56	0.06	µM
NO_2 treated	0.55	0.07	0.58	0.06	µM
NH_4 reference	5.41	5.60	7.68	7.42	µM
NH_4 treated	2.54	1.51	2.13	1.99	µM
PO_4 reference	0.18	0.10	0.21	0.11	µM
PO_4 treated	0.14	0.06	0.07	0.03	µM
TSS-reference	54.1	14.4	13.1	6.6	mg/L
TSS-treated	22.9	4.6	10.4	1.4	mg/L
POC -reference	10.8	2.2	5.2	1.4	mg/L
POC-treated	7.2	1.8	4.9	0.7	mg/L
DOC –reference	2.2	0.5	1.6	1.1	mg-C/L
DOC-treated	2.2	0.5	2.1	0.5	mg-C/L

Table 6 clearly indicates that, with exception of the values for DOC, the concentrations and values of the basic parameters were in accordance with the criteria as indicated in the guidelines (G8, Table 1). As far as the oxygen values of the reference tanks are concerned values at intake were closely corresponding to or higher than the saturation values (100%) for the given salinity and temperature. Since the water temperature increased steadily during the test series and oxygen values are presented as a range and not averaged (temperature effect on concentration). As expected on day 5 (time of discharge), the oxygen concentration in the reference tank declined by as much as 26% compared to intake values. The oxygen concentration in the treated tank was slightly less affected, most likely due to reduced microbial (bacterial) activity. As a result the oxygen saturation percentage declined only 15% relative to the value at intake.

The average concentration of the total suspended solids (TSS) was in accordance with the guidelines but varied, by a factor of two, for the individual test cycles. The particulate organic carbon (POC) concentrations were on average two times higher than the recommended values in the G8 Guidelines at intake. At discharge sediment concentration declined largely, mainly because of sedimentation in the storage tank during the holding period of 5 days. The applied filtration step (effective mesh size of 20 micron) reduced the amount of TSS and POC at intake, on average by as much as 58% and 33%, respectively. The remaining suspended material remained in suspension at discharge and the second filtration step, at discharge, did not significantly reduced TSS or POC concentration further.

The ambient DOC concentration of the challenge water at intake was circa half of the recommended concentration in the G8 guidelines and no additions were made to increase this value. Considering the much higher POC values the total organic load is considered to be high enough to compensate this lower DOC value. Furthermore, the amount of dissolved organic carbon (DOC) was hardly affected by the ballast water treatment system at intake and discharge.
With respect to the concentration of inorganic nutrients (NO_3, NO_2, NH_4 and PO_4) there were no significant differences between the values measured in the reference and treated ballast water tank. Even the NO_2 concentration of which it is known that in presence of nitrate UV-photolysis produces this by-product (Anonymous, 2010; Buchanan et al. 2006) showed no significant increase immediately after intake. For comparison the observed concentrations of nitrite were lower than the US (EPA) and EU maximum allowed concentration for drinking water of 71.4 and 7.9 µM (1000 µg-N/L and 110 µg-N/L), respectively (Sharpless et al. 2003).

High salinity

Table 7: average or range of oxygen, pH, TSS, inorganic nutrients, POC and DOC concentrations of test series run at high salinity (6 test runs in total; IX-XIV) at intake and discharge; [1]: relative to saturation value for given salinity and temperature. In the case of treated water after the treatment system.

Parameter	Intake	s.d.	Discharge (day 5)	s.d.	unit
O_2 reference[1]	101-105	-	74-89	-	%
O_2 treated[1]	96-112	-	75-97	-	%
pH reference	8.07	0.02	8.10	0.19	
pH-treated	8.22	0.10	8.16	0.13	
NO_3 reference	13.52	3.28	8.30	7.02	µM
NO_3 treated	14.56	2.27	17.95	7.65	µM
NO_2 reference	0.46	0.17	0.26	0.21	µM
NO_2 treated	0.47	0.14	0.52	0.14	µM
NH_4 reference	3.15	1.69	3.24	2.55	µM
NH_4 treated	3.30	1.56	3.52	1.55	µM
PO_4 reference	0.17	0.09	0.19	0.12	µM
PO_4 treated	0.22	0.11	0.51	0.44	µM
TSS-reference	25.9	10.8	8.3	2.8	mg/L
TSS-treated	21.5	9.4	12.1	3.0	mg/L
POC -reference	8.2	2.8	3.9	1.2	mg/L
POC-treated	7.2	2.3	5.9	1.5	mg/L
DOC –reference	2.6	0.3	2.5	0.3	mg-C/L
DOC-treated	2.8	0.4	2.5	0.2	mg-C/L

The results of the basic parameters, in terms of differences between reference and treated, at the high salinity test cycle showed comparable results as for the low salinity range (Table 7). At intake the oxygen concentrations were lower than observed for the low salinity test runs. This was mainly due to the temperature increase of the water in

the Wadden Sea. Nevertheless, the ambient water was nearly saturated with oxygen. At discharge oxygen concentrations declined considerably, but this was mainly due to microbial activity (respiration). At discharge the oxygen concentration was certainly not depleted and would not cause (regional) anoxic conditions. Partly because of the increase in seawater from the open North Sea sediment, POC and DOC values were lower as compared to the low salinity test series but still greatly higher than the minimum requirements according to the G8 guidelines. The filtration step turned out to be an effective manner of reducing the particle load but far less effective than during the low salinity test runs. TSS content and the POC content declined by 16 % and 13%, respectively.
Other differences were in the concentrations of the inorganic nutrients and in particular nitrogen. The nitrate, nitrite and ammonia concentrations were all lower at intake as compared with the low salinity test runs.

8.2 Biology

Organisms in size class > 50 μm

For the land based tests natural plankton was used and the required diversity of organisms (5 species of at least 3 different phyla) was easily fulfilled for all tests (Table 8). On average more than 10 different species of at least 5 different phyla were present in each sample (full details on species present see Annex 1).
Regarding the required minimal numbers of organisms per volume (Table 2), the values for both salinity series were for all individual tests well above the minimum requirements. On average numbers in the >50 μm size fraction were 8.3 times higher than the minimum required number of 100,000 m^{-3} at the low salinity test cycles. At the high salinity range the water at intake was on average 3.7 times above the minimum numbers of organisms. It should be noted that was exceptional and partly due to the ongoing presence of the colony forming phytoplankton species *Phaeocystis globosa*.
On discharge the number of organisms had declined dramatically in the treated tank, to an average value for the low and high salinity test series of 2.43 and 2.61 organisms m^{-3}, respectively.
During the combination test run V &VI the number of organisms in the treated water was unexpectedly high (table 8) already at intake (12.3 and 17.0). In the case of test run VI this resulted also at discharge in a number of organisms exceeding the maximum of the D2-Standard (11.7). After inspection of the BWT system is was clear that this was due to a damaged sealing causing a leakage. This sealing was replaced after discharge (T5). Despite the fact that first five test cycles on a row showed valid results two additional test runs were conducted with a new installed sealing (VII & VIII) to ensure reproducibility of the system. For this reason the results of test run VI were excluded from the data set of the fraction of organisms > 50 micron in minimum dimension (Table 8). It should be noted that this was only done for the larger sized organisms since there was no notable effect measured on the other size classes of organisms, hence results of biological parameters of test run VI was included in all other data sets.
A comparison of the number of organisms at intake versus discharge showed that the applied 20 micron primary filter was very effective in reducing the number of organisms. While there were virtually no organisms present in the treated water numbers of organisms in the reference tank were still significantly above the G8 requirement and visual inspection and viability measurement indicated that these organisms were both intact and viable.

Table 8: Number of organisms (> 50 µm) of individual test runs and averages for the salinity range at intake (T0) and on discharge at day 5 (T5) of the reference (C) and treated (ATC) tank. All numbers are presented per m^3. [1]excluding test run VI.

total plankton > 50 µm	low salinity			numbers/m^3
	Intake	Reference	Treated	
test run	C-T0	C-T5	ATC-T0	ATC-T5
I	2.61E+5	0.15E+4	0	0.3
II			1.3	0.3
III	13.4E+5	4.24E+4	0	1.3
IV			0.7	3.0
V	1.24E+5	2.73E+4	12.3	6.3
VI			17.0	11.7
VII	15.9E+5	1.49E+4	0	0.3
VIII			0	5.3
average	8.30E+5	2.15E+4	2.05[1/]3.92	2.43[1/]3.58
s.d.	7.46E+5	1.74E+4	4.56[1/]6.77	2.53[1/]4.02

total plankton > 50 micron high salinity				numbers/m^3
	Intake	Reference	Treated	
test run	C-T0	C-T5	ATC-T0	ATC-T5
IX	14.9E+5	3.35E+4	0	0.7
X			0	4.7
XI	4.41E+5	3.84E+4	0	1.7
XII			1.0	7.3
XIII	1.68E+5	3.52E+4	0.3	0.7
XIV			0	0.7
average	3.66E+5	3.57E+4	0.22	2.61
s.d.	6.13E+5	2.51E+3	0.43	2.92

Organisms in size class of 10 – 50 µm

This size class was dominated by phytoplankton, although heterotrophic organisms like ciliates and flagellates were occasionally present in high numbers at intake. Additional studies indicated that even gentle pumping of water into the reference tank resulted in a substantial damage of the fragile fraction of the plankton community like flagellates and ciliates. For that reason the flow cytometric analysis included chlorophyll *a* autofluorescence as a primary selection parameter. Furthermore, the photosynthetic efficiency of the phytoplankton community was measured, as a tool to determine the efficacy of the treatment more specifically, on this group of organisms. Finally, a 10 L subsample of both the reference and treated water was collected at intake and for the treated water also after the second UV-irradiance disinfection at discharge and

incubated under optimal growth conditions for a period of 5 days. Results of (phyto)plankton numbers in the 10 – 50 μm size range of both reference and treated incubations are included in the table for comparison with the actual measurement of the tanks (Table 9).
Table 9 shows that regarding the numerical abundance of organisms at intake for both salinity regimes the test water, with exception of the combined test run XIII/XIV, contained sufficient (phyto)plankton numbers according to the Guidelines G8.
Prolonged holding of the phytoplankton community in the 'ballast water'tank in the dark (5 days) resulted, as expected, in a considerable reduction in the number of cells at discharge on average by a factor 10 (reference tank). Although on average the plankton community of the reference tanks still contained sufficient numbers of viable organisms to meet the G8 requirements for discharge in two occasion numbers dropped to a value of slightly less than the minimum of 100 cells/mL (test runs XI and XIII). On the other hand, the bottle incubations (under optimal growth conditions), which were run parallel to the large tank incubations (C-inc-T5), showed an extensive increase in numerical abundance of (phyto)plankton within the five days holding period. This high growth response was clearly indicative of the presence of a viable population of mainly phytoplankton.

Figure 12: internal view of Aqua TriComb™ system showing the prefilter (top) and UV-reactors (bottom).

In contrast, treatment with the UV-reactor resulted in instantaneous die-off of phytoplankton. Total cell numbers declined by a factor two immediately after treatment (ATC-T0). This implies that in terms of numerical abundance the total count of intact cells remained fairly high after their first passage through the UV-reactor (T0). After a holding period of five days in the tanks residual intact and chlorophyll containing phytoplankton cells were still detected flow cytometric but numbers dropped by a factor 16 (low salinity) and 4.6 (high salinity), respectively. However, analysis of the photosynthetic efficiency of the phytoplankton cells (Table 11) clearly demonstrated that these cells were no longer viable. The incubated experiments confirmed the low abundance of intact cells (ATC-inc-T5) and on average the number of viable cells after the first treatment step was <10 cell/mL. Because the ATC™ BWT system also included a second passage through the UV-reactor at discharge this water was also incubated for a period of five to seven days to determine the efficacy of this second passage. Flow cytometric data showed that at the high salinity test cycle the total number of cells declined further, from 6.2 down to 1.5 cells/mL. In contrast at low salinity there was a slight increase in the number of intact cells, from 3.9 to 13.6 cells/mL). This was not due to a general trend but observed incidentally at the test runs IV and V.

Table 9: Total plankton number of plankton in the 10 – 50 µm size range at intake (WS= Wadden Sea), reference (C) and treated tank (ATC), reference and treated incubated samples of the intake (Inc.) at day 5 (T5) during discharge and of the incubated discharge samples (Dis-T12). Runs I to VIII at low salinity range and IX to XIV at high salinity range. All numbers are presented per mL as total counts or as viable cells (*). T12= 12 days after intake.

10- 50 µm cell numbers cells/ml									ATC
low salinity	Intake		Reference		Treated	viable		viable	
test run	WS	C-T5	C-inc-T5	ATC - T0	ATC -T5	ATC - T5*	ATC -inc -T5	ATC -inc -5*	ATC -Dis -T12
I	1237	204	4637	411	33	<10	0.0	<1	1.6
II				1140	43	<10	0.0	<1	2.4
III	1897	124	13864	425	4.0	<10	10	<1	13
IV				261	18	<10	2.4	<1	40
V	1021	113	14632	1167	9	<10	13	<1	40
VI				1125	23	<10	6.3	<1	7.0
VII	1408	110	8626	803	125	<10	0.0	<1	4.0
VIII				373	98	<10	0.0	<1	0.0
average	1391	138	10439	713	44	<10	3.9	<1	13.6
high salinity									
IX	1321	120	5839	436	267	<10	1.6	<1	0.0
X				219	98	<10	0.0	<1	0.0
XI	1650	93	4451	1116	179	<10	6.3	<1	2.4
XII				1274	93	<10	15	<1	3.2
XIII	714	87	1616	448	112	<10	5.6	<1	1.6
XIV				449	92	<10	8.7	<1	1.6
average	1228	100	3969	657	140	<10	6.2	<1	1.5

In order to have a more comprehensive insight of the fate of all organisms, i.e. also the planktonic fraction < 10 µm in size diameter were monitored in the control and treated water (Table 10). A dominant species of phytoplankton in the size range of ca. 6 µm in cell diameter (*Phaeocystis globosa)* was used for this purpose. Although no clear criteria currently are defined for this size class it was clear that the Aqua TriComb™ BWT system was also effective in reducing organisms in this size range. In general the response of *Phaeocystis* in terms of numbers and viable cells showed that treatment (ATC-T5) was as effective in reducing the number of cells of this group of phytoplankton as compared to the plankton in the 10 to 50 µm size class (Table 9). Applying flow cytometry showed the presence of intact phytoplankton in the treated tanks even after five days. Viability tests indicated that these remaining cells were non-viable. This was also demonstrated in the incubation experiments. Incubating the treated water at discharge, for 5 to 7 days, showed a slight increase in numbers of *Phaeocystis* cells at the low salinity test runs (three fold increase).

Table 10: number of a dominant phytoplankton species present (*Phaeocystis globosa*; ca. 6 µm in cell diameter) at each test run and average for the salinity range at intake (T0), reference (C) and treated tank (ATC), reference and treated incubated samples of the intake (Inc.) at day 5 (T5) during discharge and of the incubated discharge samples (Dis-T12). Test runs I to VIII at low salinity range and IX to XIV at high salinity range. All numbers are presented per mL as total counts or as viable cells (*). T12= 12 days after intake.

***Phaeocystis* ~ 6 µm cells/ml**									**ATC**
low salinity	**intake**	**Reference**			**Treated**		**viable**		**viable**
test run	**C-T0**	**C-T5**	**C-inc -T5**	**ATC -T0**	**ATC -T5**	**ATC - inc -T5**	**ATC -inc -T5***	**ATC -Dis -Tx**	**ATC -Dis -T12***
I	2118	160	1317	1103	4.0	n.d.	<1	0.8	<1
II				1301	1.0	n.d.	<1	1.6	<1
III	4310	544	6074	2196	30	5.6	<10	228	<10
IV				2118	111	13	<10	202	<10
V	5364	979	7966	4385	12	23	<10	200	<10
VI				2387	13	2.4	<10	98	<10
VII	8314	339	9564	1240	72	1.6	<10	126	<10
VIII				948	40	0.8	<10	14	<10
average	5026	505	6230	1960	35	5.9	<10	109	<10
high salinity									
IX	9529	552	12266	8408	2438	0.8	<1	0.8	<1
X				5345	755	0.8	<1	0.0	<1
XI	3617	950	4653	2968	330	4.8	<10	24	<10
XII				3604	188	4.0	<10	0.0	<1
XIIII	1943	472	1570	1332	103	1.6	<10	0.8	<1
XIV				1302	81	0.8	<10	n.d.	<1
average	5029	658	6163	3826	649	2.1	<10	4.2	<1

Photosynthetic efficiency

Another approach to gain insight in the physiological condition and therefore the growth potential of the phytoplankton community is by measuring the photosynthetic efficiency

(F_v/F_m) of the phytoplankton. This was done for the whole community (entire phytoplankton fraction with cell diameter of <50 µm), but when required these measurements were also repeated for different size fractions. For a clear understanding it must be noted that values of F_v/F_m > 0.4 are indicative of a healthy phytoplankton population; an F_v/F_m < 0.4 indicates that the phytoplankton community is experiencing severe stress, and a value < 0.1 is typically observed in decaying phytoplankton populations (terminal stage).

Table 11 shows that during intake the whole phytoplankton community of the Wadden Sea (low salinity range) and coastal waters of the North Sea (high salinity range) was physiologically in a (very) healthy condition, i.e. containing mostly photosynthetic active and therefore viable cells. Only at the first test run (I & II) the phytoplankton community at intake (C-T0) showed signs of a reduced viability. However, after transferring this population to optimal growth conditions there was a complete recovery. Prolonged incubation in the dark (for phytoplankton prolonged darkness of five days is to be considered a major stress factor) of the phytoplankton community in the reference tanks resulted in a severe reduction in the photosynthetic efficiency. With exception of the first test run on average a decline by a factor two. This trend coincided with the major decline in cell numbers measured after this five days dark period (Tables 9 and 10). In contrast the photosynthetic efficiency of the phytoplankton from the same water mass but incubated under optimal growth conditions showed that the cells maintained a healthy status (C-inc-T5). In the presence of excess nutrient this high viability resulted in an increase in numerical abundance (Tables 9 and 10).

After the first treatment, at intake (ATC-T0), the physiological condition of the phytoplankton community was typical of a population under severe stress, resulting in F_v/F_m values of <0.2 (ATC-T0). After a holding time of five days in the (dark) storage tank the photosynthetic efficiency was further reduced to a value corresponding with a decaying population. In the samples, incubated under optimal growth conditions, no recovery of the phytoplankton population was observed within the time span of five days. The second UV- disinfection step did not alter the Fv/Fm value from the first UV-disinfection step (ATC-T5).

Table 11: Photosynthetic efficiency (F_v/F_m) of the whole phytoplankton community (2 - > 50 µm cell diameter) at each test run and average values for the salinity range at intake (T0), reference (C) and treated tank (ATC), reference and treated incubated samples of the intake (Inc.) at day 5 (T5) during discharge and of the incubated discharge samples (Dis-T12). Test runs I to VIII at low salinity range and IX to XIV at high salinity range. T12= 12 days after intake.

F_v/F_m	Intake	Reference		Treated			
low salinity	C-T0	C-T5	C-inc -T5	ATC -T0	ATC -T5	ATC -inc-T5	ATC-Dis-T12
I	0.21	0.22	0.54	0.19	0.02	0.06	0.18
II				0.14	0.01	0.03	0.03
III	0.61	0.25	0.53	0.19	0.00	0.08	0.00
IV				0.07	0.01	0.03	0.00
V	0.63	0.29	0.62	0.00	0.02	0.04	0.00
VI				0.00	0.02	0.00	0.00
VII	0.60	0.23	0.56	0.10	0.01	0.01	0.00
VIII				0.02	0.05	0.11	0.00
average	0.51	0.25	0.56	0.09	0.02	0.04	0.03
s.d.	0.21	0.03	0.04	0.08	0.01	0.04	0.06

Table 11 continued							
high salinity							
IX	0.52	0.22	0.63	0.03	0.01	0.05	0.20
X				0.03	0.02	0.00	0.17
XI	0.61	0.29	0.52	0.05	0.03	0.03	0.19
XII				0.19	0.03	0.06	0.20
XIII	0.60	0.29	0.25	0.12	0.04	0.10	0.10
XIV				0.05	0.06	0.14	0.06
average	0.58	0.27	0.47	0.13	0.03	0.06	0.16
s.d.	0.05	0.04	0.20	0.18	0.02	0.05	0.06

Bacteria

For the microbial community the presence/absence of two types of human pathogens was monitored prior to (intake, T0) and after treatment (T5), while the response of the whole microbial community was also assessed (total bacteria).

Table 12 shows that even during intake the number of the target microorganisms was well below the standard as indicated in the D2-Standard. The reason for this is that the NIOZ harbour is located in a pristine environment with little or no urban activity. Subsequently, also the number of human pathogens in the reference and treated tanks were below detection limit during discharge.

Table 12: plate counts of the human pathogens *E. coli* (cfu per mL) and total enterococci (cfu per 100 mL) of each test run and averages for the salinity range at intake (T0), reference (C) and treated tank (ATCS), reference and treated incubated samples of the intake (Inc.) at day 5 (T5) during discharge and of the incubated discharge samples (Dis-T12). All numbers are presented per mL as total counts or as viable cells. T12= 12 days after intake.

human pathogens	**Intake**		**Reference**		**Treated**			
counts	**C-T0**	**C-T0**	**C-T5**	**C-T5**	**ATC -T5**	**ATC -T5**	**ATC-Dis-T12**	**ATC-Dis-T12**
low salinity	*E.coli*	Enterococci	*E.coli*	Enterococci	*E.coli*	Enterococci	*E.coli*	Enterococci
I	<0.1	<1	<0.1	<1	nd	<1	<0.1	<1
II					<0.1	<1	<0.1	<1
III	<0.1	<1	<0.1	<1	<0.1	<1	<0.1	<1
IV					<0.1	<1	<0.1	<1
V	<0.1	<1	<0.1	<1	<0.1	<1	<0.1	<1
VI					<0.1	<1	<0.1	<1
VII	<0.1		<0.1	<1	<0.1	<1	<0.1	<1
VIII					<0.1	<1	<0.1	<1
range	<0.1	<1	<0.1	<1	<0.1	<1	<0.1	<1
high salinity								
IX	0.1	<1	<0.1	<1	<0.1	<1	<0.1	<1
X					<0.1	<1	<0.1	<1
XI	<0.1	<1	<0.1	<1	<0.1	<1	<0.1	<1
XII					<0.1	<1	<0.1	<1
XIII	<0.1	<1	<0.1	<1	<0.1	<1	<0.1	<1
XIV					<0.1	<1	<0.1	<1
range	<0.1-0.1	<1	<0.1	<1	<0.1	<1	<0.1	<1

In contrast to the near absence of human pathogens, the typical marine microbial community was abundantly present. The total bacterial community is well studied in the Wadden Sea, using a method based on staining the nucleic acid of the cells during the past decade. The currently observed numbers ranging from 3.6 to 11 * 10^6 cells per mL (variation of factor 3) are numbers typically observed in spring and early summer situation in the area (Table 13). Of this total bacterial population only a very small fraction (<0.1%) could be identified as heterotrophic ones using the classical plate assay method (39 to 1500 cfu's per mL). Only at one test run (V & VI) a value of 1500 cfu's was measured. Therefore, if only plating would be used as criteria for heterotrophic bacteria number this would result in a severe underestimation of the actual bacteria numbers. After 5 days of incubation total bacteria numbers in the reference tank declined, by as much as 53% in the control tank but there was considerable variation between the different test runs (C-T5). At one test run (VII & VIII) there was a slight increase in the bacteria numbers.

Bacteria numbers in the incubated water of the reference tanks (C-inc-T5) showed a decline as well as a numerical increase in numbers relative to the numbers measured at intake (T0).

In the treated tanks, immediately after the first UV- disinfection step, there was not a significant loss in terms of bacteria numbers. After five days of incubation numbers dropped roughly by 40% (ATC-T5). Incubating the treated water under, for phytoplankton, optimal growth conditions showed numbers of bacteria which were only slightly higher than those observed in the standard reference tank. A second passage through the UV- reactor and after a delay of five to seven days (ATC-Dis-Tx) resulted in significant loss in intact cells by as much as 70% as compared to the bacteria counts at intake.

In terms of cultivable heterotrophic bacteria the treated water (single passage through the UV-reactor) was favourable to the heterotrophic bacteria as up to 10 fold higher numbers were found relative to the intake values from the Wadden Sea. The second UV-treatment did not change this trend. In particular that the high salinity test cycle the number of heterotrophic bacteria increased proportionally, resulting in a > 100 fold increase in the number of cfu's.

Table 13: Total bacteria number (numbers per mL) and colony forming units of heterotrophic bacteria (colony = cfu numbers per mL) of each test run and average for the salinity range at intake (T0), reference (C) and treated tank (ATC), reference and treated incubated samples of the intake (Inc.) at day 5 (T5) during discharge and of the incubated discharge samples (Dis-T12). All numbers are presented per mL as total counts or as viable cells. T12= 12 days after intake. nd: not determined

bacteria	Intake	Reference				Treated					ATC
	C-T0	C-T0	C-T5	C-T5	C-inc-T5	ATC -T0	ATC -T5	ATC-T5-Inc	ATC -T5	ATC-Dis-T12	ATC-Dis-T12
low salinity	total bact.	cfu	total bact.	cfu	total bact.	total bact.	total bact.	total bact.	cfu	total bact.	cfu
I	3.61E+6	270	2.56E+6	220	4.19E+6	3.41E+6	2.80E+6	3.34E+6	880	1.87E+6	200
II					2.87E+6	3.47E+6	3.65E+6	3.93E+6	2100	2.65E+6	100
III	6.37E+6	220	2.37E+6	210	4.77E+6	4.29E+6	2.49E+6	3.93E+6	1200	1.08E+6	170
IV						5.47E+6	3.21E+6	3.41E+6	300	1.46E+6	1200
V	5.49E+6	1500	4.53E+6	250	5.08E+6	4.89E+6	2.07E+6	4.46E+6	490	2.48E+6	1800
VI						5.19E+6	2.91E+6	4.06E+6	770	2.66E+6	80
VII	7.78E+6	39	8.19E+6	420	8.58E+6	7.06E+6	3.20E+6	4.32E+6	980	1.63E+6	280
VIII						8.02E+6	5.07E+6	5.93E+6	260	1.43E+6	96
average/ range	5.81E+6	39-1500	4.41E+6	270	5.10E+6	5.22E+6	3.17E+6	4.17E+6	290-2100	1.91E+6	80-1800
high	salinity										
IX	6.05E+6	40	3.17E+6	2	10.3E+6	6.13E+6	3.98E+6	8.20E+6	>3000	4.07E+6	>3000
X						5.54E+6	2.80E+6	6.66E+6	130	1.84E+6	>300
XI	10.7E+6	7	4.10E+6	15	3.79E+6	10.0E+6	3.24E+6	6.30E+6	200	2.04E+6	1400
XII						9.77E+6	6.83E+6	5.00E+6	30	3.36E+6	>3000
XIII	7.12E+6	<1	3.36E+6	3	5.59E+6	6.22E+6	2.39E+6	3.82E+6	18	1.96E+6	1400
XIV						6.09E+6	2.60E+6	4.99E+6	530	1.42E+6	1600
average/ range	7.96E+6	<1-40	3.55 E+6	7	6.55E+6	7.30E+6	3.64E+6	5.83E+6	18->3000	2.45E+6	1400->3000

9 Environmental acceptability

Ballast water treatment systems applying active substances, but also systems that do not use active substances should demonstrate according to the revised guidelines G8 (MEPC 174.58, Anonymous 2008) that the treated water upon discharge is not harmful to the environment and organisms.

Although officially not obligatory for the Aqua TriComb™ BWT system a series of studies were conducted to examine long-term (25 days) potential regrowth of organisms and the vitality of the UV-irradiated ballast water. In total 2 long-term incubation experiments, one at each salinity regime, were conducted with treated water which was collected in an ultraclean container (Nalgene 10 L) and incubated under optimal growth conditions for phytoplankton to allow in particulate germination of resting stages and cysts (Figure 13). For comparison the long term fate on the remaining phytoplankton was also monitored in a parallel experiment but in this case the incubation container was kept in the dark throughout the whole period (Figure 14).The experiments were done for treated water collected immediately at intake (pre-filtration and single passage through the UV-reactor) and at discharge (second passage through the UV-reactor). The second series of (discharge) samples had already experienced five days of darkness in the large storage tank (200 m^3) prior to the incubation.

To stimulate regrowth of planktonic organism extra nutrients were added (nitrate; 30 µM, phosphate; 2 µM, silicate; 20 µM, final concentration). In particular at the peak of the spring bloom and thereafter the ambient nutrient concentration (mainly silicate and phosphate) can be extremely low, which may prevent growth of phytoplankton and also bacteria.

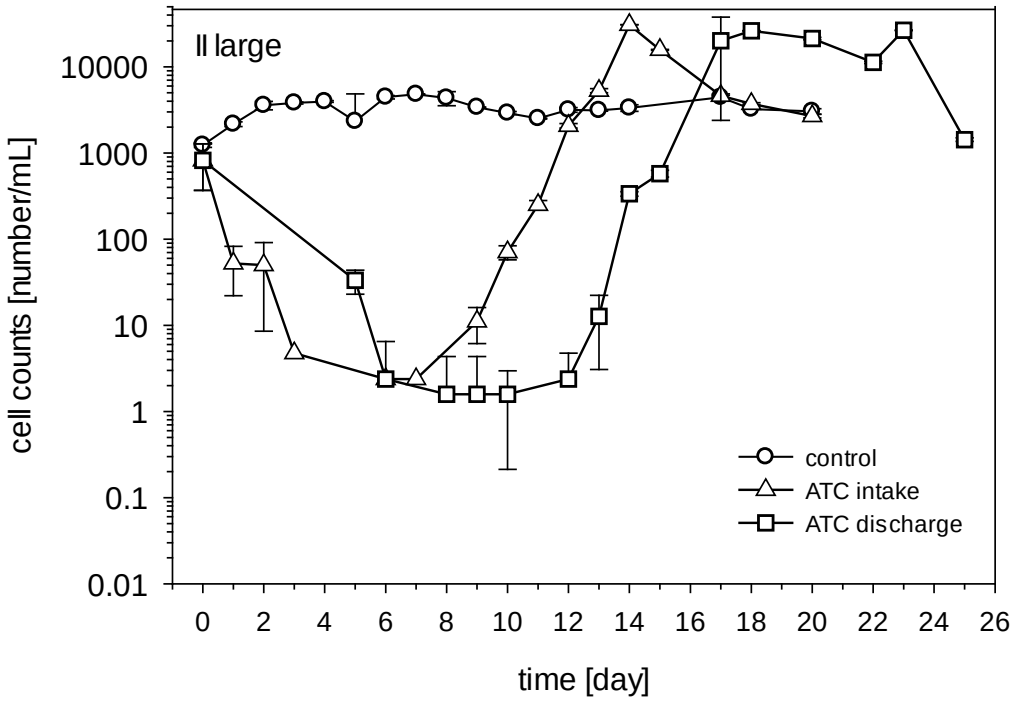

Figure 13: time course of total phytoplankton numbers (10 – 50 µm size range) during a 25 day incubation period (light), control and treated water incubated at intake and treated water also incubated at discharge. Test run II; values are mean of three replicate samples. Numbers are in log scale.

The phytoplankton community in the size class of 10-50 µm, present in the water collected from the reference tank and incubated under optimal growth conditions showed an instantaneous growth response (Figure 13). Numbers increased by a factor of

four in the first three days of the incubation. In contrast phytoplankton cell numbers in the treated water at intake showed a gradual decline during the first six days. After day six (exponential) regrowth of phytoplankton occurred. This observation indicates that a single UV-disinfection treatment did not deactivate the entire plankton community. Even as few as two (viable) cells per mL did recover from the UV-irradiance disinfection step. Although figure 13 only shows the results for the larger fraction a similar trend was observed for the smaller phytoplankton as well (data not shown).
For this reasons the Aqua TriComb™ BWT system was applying a second UV- disinfection step at discharge (day 5). This resulted in a further, but slight reduction of the number of phytoplankton as much as a factor 10. However, also after two UV-disinfection cycles regrowth was observed from day 12 onwards after the initial intake of the water. The second UV- disinfection step was certainly effective in this respect but even this twice disinfected water was still not entirely free of living plankton on the long-term.

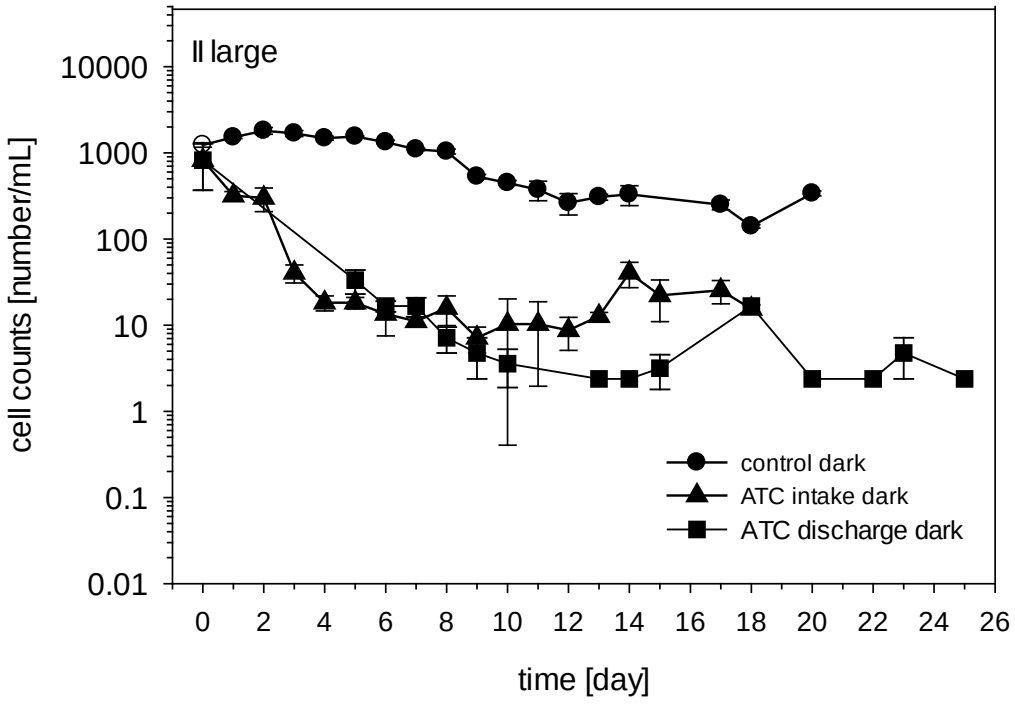

Figure 14: time course of total phytoplankton numbers (10 – 50 µm size range) during a 25 day (dark) incubation period, control and treated water incubated at intake and treated water also incubated at discharge. Test run II; values are mean of three replicates. Numbers are in log scale.

Repeating the same incubation and treatment procedures but keeping the water samples in the dark instead of incubating the sample bottles in the light showed in all three bottles a gradual decline in cells numbers over time. In the control cell numbers remained constant during a period of up to 6 days, gradually declining thereafter. In the both bottles containing UV-disinfected water phytoplankton numbers declined rapidly, by several orders of magnitude in the first 10 days but remain constant thereafter.

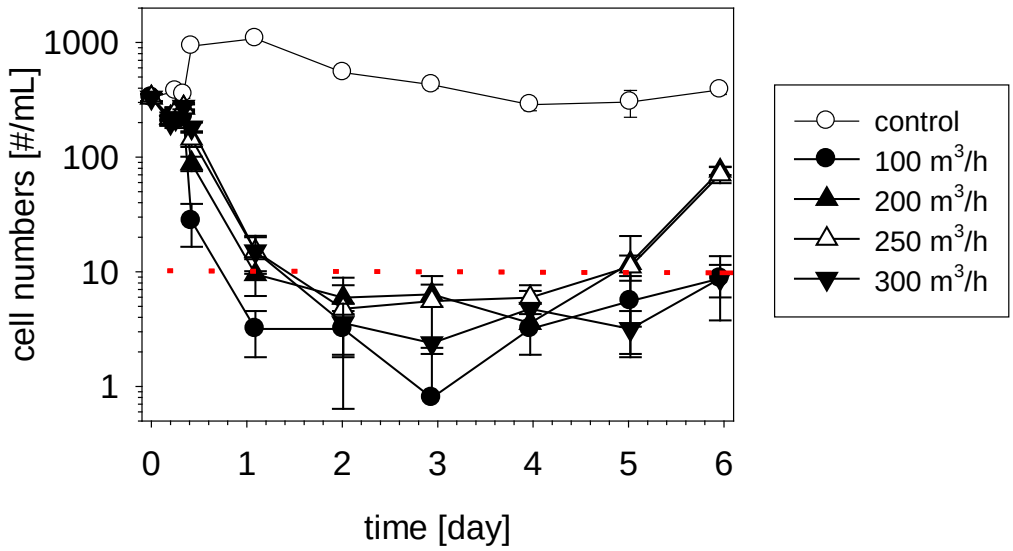

Figure 15: time course of total phytoplankton numbers (10 – 50 µm size range) during a 6 day incubation period, control and treated water incubated at intake with constant UV-radiation but varying flow rates. Typical standard flow rate was 200 m^3/h. Values are mean of three replicates. Red dotted line indicates D2-Standard of 10 cells per mL. numbers are in log scale.

In order to study the delayed response of UV-radiation on the mortality and survival rate of phytoplankton the challenge water was treated at four different UV-dosages (constant UV-irradiation but varying flow rates). The numbers of intact phytoplankton cells were determined with a high sampling frequency immediately after passing the UV-reactor (Fig. 15). In contrast to the control at all UV-light intensities tested a significant decline in number, indicative of ongoing mortality, was observed for of up to 3 days. The D2-Standard was nevertheless reached already after one day. In general the highest dosage, i.e. lowest flow rate (100 m^3/h) showed the strongest response in terms of cell mortality. The differences between the three other flow rates were only minimal.

Figure 15 only presents the data for the larger fraction (10 – 50 µm) of the phytoplankton but a similar trend was observed for the smaller size phytoplankton cells as well.

In terms of vitality it should be noted that regrowth of phytoplankton was observed even under a four times higher UV-dosage as standard applied (Figure 14). Therefore, potential by- or end-products generated by the UV- reactor did not affect the water quality in such a manner that growth of phytoplankton in the receiving environment would be inhibited.

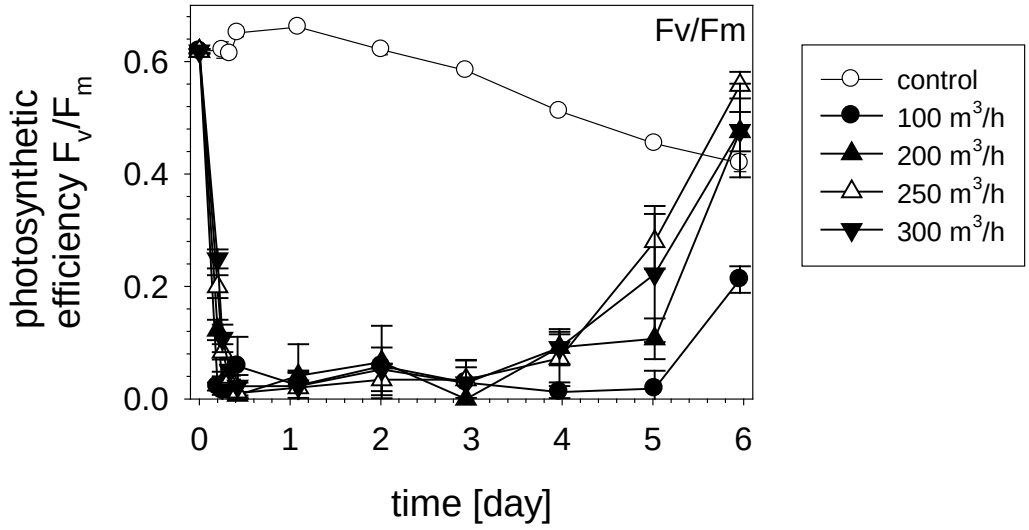

Figure 16: time course of the photosynthetic efficiency (Fv/Fm) of the whole phytoplankton community (2 to 50 µm) during a six day incubation period. Conditions identical to figure 15.

With respect to photosynthetic efficiency of the phytoplankton community values drop immediately, within few hours, to a value of less than 0.1 which is typical for a decaying population. Nevertheless, recovery occurred after day four. Of all four treatments the one with the highest dosage (lowest flow rate) showed the least recovery.

10 Discussion and evaluation of results

The presented data show that the experimental design, type of test protocols used and additional experiments provide a solid data base of information on the performance of the Aqua TriComb™BWT system under semi-*in situ* conditions. The total time span of testing covered the entire spring and early summer season, including a large variety in terms of organism composition. The combined effect of a fine mesh filter (20 µm) and multiple UV-reactors was capable of reducing numbers of organism below the D2-Standard.

With only some minor deviations the present 14 test runs were conducted according to the guidelines G8 of the IMO. The basic chemistry and physical properties of the water were not altered by the treatment and no identifiable factors could be detected affecting the vitality of the treated water upon discharge.

The treated water remained stable for most of the parameters over the whole holding period of 5 days. Concentrations of dissolved oxygen (DO) and dissolved organic carbon (DOC) hardly varied as compared to the reference water. Only the concentration of total suspended solids and the particulate organic carbon declined slightly mainly as a result of sedimentation. In this respect it should be noted that the application of the self-cleaning filter removed larger particles and the load of sediment in the treated tanks was only minimal compared to that in the control tank. In one occasion a leakage of the sealing of the filter was observed (combined test run V and VI). After repair an extra set of tests was conducted to confirm a proper repair.

With respect to the water chemistry the currently applied configuration did not result in an increased nitrite concentration, as has been reported to associated with some UV-radiation reactors (cf. Sharpless & Linden 2001).

The stable DO and DOC over time was indicative of only minimal biological activity during the holding period of 5 days. Numbers of organisms were in compliance with the D2-Standard at discharge but the second UV-disinfection effectively reduced the number of viable organism further. The treated water did not contain toxic or growth inhibiting substances. Neither was NO_2 produced as by-products. On the contrary this water was rich in growth stimulating nutrients (nitrogen, phosphorus and silicate) for phytoplankton and bacteria.

With apparently some living cells present and ample growth stimulating resources present, phytoplankton and bacteria could start flourishing when transferred to growth stimulating conditions. Still even under these favourable conditions growth was delayed by a several days.

During the testing period we encountered some issues which need attention for future testing and legislation.

1. The long-term incubations (ca. 25 days) and the environmental acceptability studies provide good insight in the response of the whole community to the growth potential/limitation of the treated water when released into environment. These experiments should be conducted next to the standard set of toxicology and residual chemistry tests of the treated ballast water.
2. Testing for the presence of human pathogens strongly depends on the natural abundance of these microbes in the natural environment. Since these pathogens cannot be supplemented for safety reasons accurate testing is therefore not possible. Moreover, viability tests of the total bacteria community showed that not all bacteria were effectively deactivated. Therefore, at least in theory the human pathogens would remain a potential risk. This is a factor of concern for the land-based tests as well as for the ship-board trials when the ship remains in fairly clean ports for intake and discharge. Although UV systems, including the one

applied in the present BWT-system, in general have been proven to be effective in the disinfection of (drinking) water no data test are available

3. The Guidelines G8 and the update version as adopted at MEPC 174.58, (Anonymous 2008) are incomplete with respect to treatment systems including a second treatment at discharge. In line of the tank and sample volume for treatment at intake a similar protocol should be developed for treatment at discharge. However, considering the expected number of organisms at discharge and statistical errors associated with the actual counts a second sample tank to collect and store the discharge would be required with a volume two orders of magnitude larger than the present tank. As an alternative for these large volume incubations, but restricted to the smaller sized plankton, the bottle (10 L) incubations either in the dark or in a more favourable condition could be applied.
4. The present observation clearly demonstrates a delayed mortality in organisms after passing a UV-reactor. For phytoplankton this can be up to three days but similar effects are to be expected for zooplankton as well. Sampling and inspection for the presence of viable cells needs to be carried out as soon as possible after water passing a ballast water treatment unit (G8 guidelines). The presence of a delayed mortality, as observed in UV-BWT systems, will result in a severe over-estimation of the actual numbers of viable organisms present and under-estimating of the efficacy of this technology.

In conclusion, the present configuration of the Aqua TriComb™ BWT system offers a environmentally safe cleaning of the ballast water resulting in residual numbers of organisms in the assigned categories well below the Standard of the IMO Regulation-D2.

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Appendix 1

Phytoplankton Marsdiep April-July 2010 ***data by Koeman & Bijkerk***	***+: present but rare, ++: present; +++: dominant;*** ***++++: very abundant; +++++: massively present***	
group	***species name***	***relative dominance***
autotrophic flagellate	*Phaeocystis globosa* colony cell	*+++++*
	Phaeocystis globosa colonies < 50 µm	*++++*
	Phaeocystis globosa colonies > 50 µm	*+++++*
autotrophic flagellate	*Phaeocystis globosa flagellate cell*	*+++++*
diatom pennate	*Pseudonitzschia delicatissima* group	*++++*
autotrophic flagellate	Prymnesiales indet. <10 µm	*++++*
diatom centricate	*Thalassiosira* spp. 10 µm	*++++*
diatom centricate	*Chaetoceros socialis*	*++++*
autotrophic flagellate	*Hemiselmis* group	*+++*
diatom centricate	*Skeletonema "costatum"* group	*+++*
autotrophic flagellate	*Plagioselmis* group	*+++*
autotrophic flagellate	*Pyramimonas* spp. <10 µm	*++*
autotrophic flagellate	*Teleaulax acuta* group	*++*
diatom centricate	*Minutocellus* group	*++*
diatom centricate	*Thalassiosira* spp. 10-30 µm	*++*
diatom centricate	*Guinardia delicatula*	*++*
freshwater green alga	*Pediastrum* spp.	*++*
autotrophic flagellate	Prasinophyceae sp. <10 µm	*++*
hetero/auto dinoflagellate	Gymnodiniaceae sp. 10-30 µm	*++*
diatom centricate	*Leptocylindrus danicus*	*++*
diatom centricate	*Brockmanniella brockmannii*	*++*
diatom centricate	*Cerataulina pelagica*	*+*
autotrophic flagellate	autotrophic flagellate sp. (<10 µm)	*+*
diatom centricate	*Chaetoceros* spp. <10 µm solitairy cells	*+*
autotrophic flagellate	Chlorophyceae sp.	*+*
freshwater green alga	*Crucigenia* spp.	*+*
freshwater green alga	Chlorophyta fresh sp.	*+*
diatom centricate	*Skeletonema "costatum"*	*+*
heterotrophic flagellate	parasite cyst of *Ochromonas* group	*++++*
heterotrophic flagellate	heterotrophic flagellate sp. <10 µm	*++++*
heterotrophic flagellate	*Paulinella* spp.	*+++*
heterotrophic flagellate	heterotrophic flagellate sp. 10-30 µm	*++*
heterotrophic flagellate	Choanoflagellatea sp.	*++*
hetero/auto flagellate	Cryptophyceae "light" group	*++*
heterotrophic dinoflagellate	*Oxyrrhis marina*	*++*
heterotrophic dinoflagellate	*Katodinium glaucum*	*++*
heterotrophic flagellate	*Ciliophrys* group (spp.)	*++*
heterotrophic flagellate	Chrysophyceae sp.	*+*
heterotrophic flagellate	*Telonema* spp.	*+*

Species list zooplankton and some larger (atypical) phytoplankton

Appendix 1 continued

Phylum	Class	Subclass, Order, etc.	species no.	Identified genera	most likely present
Sarcomastigophora		Dinoflagellida	*2*	*Noctiluca, Protoperidinium*	
Bacillariophyceae			*3+*	*Odontella, Coscinodiscus*	
Cnidaria	Hydrozoa		*2+*	*Obelia*	
	Scyphozoa		*2*	*Aurelia, Cyanea*	
Ctenophora			*2+*		*Pleurobrachia, Beroe, Mnemiopsis*
Nemathelminthes	Rotatoria		*1+*		*Asplanchna*
	Nematoda		*1+*		
Annelida	Polychaeta		*2+*		
Arthropoda	Crustacea	Order Calanoida	*4+*	*Temora, Acartia, Centropages, Calanus and/or Pseudocalanus*	*Oithona*
		Order Harpacticoida	*2+*		*Tigriopus*
		Subclass Cirripedia	*1+*		*Semibalanus*
		Suborder Cladocera	*2*	*Podon, Evadne*	
		Subclass Malacostraca	*2+*	*Carcinus (zoea larvae)*	
Mollusca	Gastropoda		*1+*		*Littorina*
	Lamellibranchia		*2+*	*Cerastoderma*	*Mya*
Echinodermata	Ophiuroidea and/or		*2+*		*Ophiothrix, Echinocardium*
	Echinoidea				
Gnathostomata	Osteichthyes		*1*		
Minimum number of species encountered (10 phyla):			*32*		